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DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
FORT DETRICK, MARYLAND 21702-5012



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29 Aug 95

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Gary R. Gilbert

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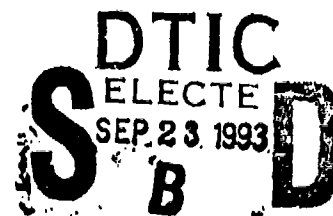
TITLE: NEUTRALIZING MONOCLONAL ANTIBODIES AGAINST BIOLOGICAL
TOXINS

PRINCIPAL INVESTIGATOR: Mark C. Glassy, Ph.D.

CONTRACTING ORGANIZATION: Hygeia Pharmaceuticals, Inc.
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REPORT DATE: August 14, 1993

TYPE OF REPORT: Phase I Final Report



PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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13. ABSTRACT (Maximum 200 words) <p>This SBIR project was broken down into two separate phases. The first phase served to demonstrate that a human immune response could be generated to the A-chain of ricin. The second phase centered on the immortalization of this immune response.</p> <p>A human immune response, though weak, to ricin A-chain was observed using IVI protocols. Different patient's spleenocytes were found to respond differently to in vitro immunization protocols with ricin A-chain as an antigen. To further improve the human immune response to rAc, different adjuvant-like compounds should be used. (e.g., rAc coupled to a highly immunogenic macromolecule).</p> <p>With the protocols used to date, the predominant immune response generated was of the IgM type. Essentially, no IgG MAb's were detected.</p>					
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FOREWORD

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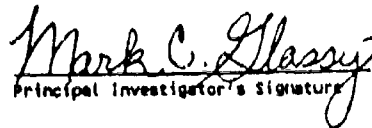
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Principal Investigator's Signature

8/14/93

Date

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INTRODUCTION

No specific treatment regime exists for a large number of biological toxins. These toxins pose a threat in a military context and also constitute a public health hazard, mainly when accidentally ingested. An important class of biological toxins are those which inhibit protein synthesis, found in plants as well as bacteria. Many of these share structural and mechanistic properties. They are proteins, often of about 60,000 MW, with two subunits, one responsible for binding to cells, the other for the toxic action. The most common targets of these toxins are in ribosomes and the most toxic of the substances kill at one molecule per cell.

HYGEIA Pharmaceuticals develops human monoclonal antibodies for use as therapeutic agents. The purpose of this study, was to begin to use ricin as a model system for the development of human monoclonal antibodies with the ability to neutralize biological toxins. The approach was to perform in vitro immunizations of human lymphocytes and to fuse these with a proprietary human fusion partner. Resulting hybridomas are to be cloned and assayed for antigen recognition and for the neutralization of ricin in model systems.

METHODS

1. Materials:

- a. human spleenocytes: these were obtained from normal donors. No specific information is available on these patients.
- b. Supernatant of pokeweed mitogen (PWM) stimulated T-cells. T-lymphocytes from normal donors were used to generate this source of growth factors. Critical for IVI protocols.
- c. Ricin A chain (rAc)

Three forms of the antigen were used.

- rAc-g.....i) glycosylated A chain (obtained from Sigma)
- rAc-ng....ii) deglycosylated A chain (obtained from Sigma)
- rAc-p.....iii) "active peptide" of rAc (obtained from Dr. Paul Lemley) (used in ELISA screens)
- d. SHFP-1: proprietary human fusion partner.
- e. Tetanus Toxin (used as internal control to verify the IVI protocol was effective).

2. Variables tested

a. Source of spleenocytes:

- i) Spleenocytes from three separate patients were analyzed. All gave similar results. (It is unknown whether additional/different patient's spleenocytes would yield similar results.)
- ii) the concentrations of spleenocytes used were varied, with no significant difference observable. We used the following in 96-well plates.

1 x 10⁵ cells/well - low
2 x 10⁵ cells/well - medium
5 x 10⁵ cells/well - high cell density

b. rAc concentration

<u>stock</u>	<u>final concentration</u>
1 ug/ml	100 ng antigen
5 ug/ml	500 ng antigen
10 ug/ml	1 ug antigen

50 ug/ml
100 ug/ml

5 ug antigen
10 ug antigen

*100 ul of the stock rAc was added per well

*amount of rAc per well of 96-well plate

c. PWN-T cell supernatant (PWM = pokeweed mitogen)

o T-lymphocytes from two separate patients were obtained and incubated with PWM.

o harvested supernatants were aliquoted and stored at -70° C.

two concentrations were used:

25% V/V

50% V/V

d. days in culture

o IVI protocols are dependent upon the time of exposure to all the critical components.

o we analyzed the IVI human immune response over a 4 week period.

o results suggest that optimizing 1) cell viability and 2) effective immune response is maximal at 6 days after initiating culture conditions.

RESULTS

The first objective of this project was to test the feasibility of using current in vitro immunization (IVI) procedures, as applied to human monoclonal antibody technology, to generate a human immune response to the ricin toxin molecule. The second objective was to successfully immortalize the human immune response to generate HuMAbs to ricin A chain.

Experimental conditions:

- 1) Condition A
 - a) 50% PWM-T spnt
 - b) [rAc-g] 100ng, 500ng, 1ug, 5ug, 10ug
 - c) 6 day incubation
- 2) Condition B
 - a) 50% PWM-T
 - b) [rAc-ng] 100ng, 500ng, 1ug, 5ug, 10ug
 - c) 6 day incubation
- 3) Condition C
 - a) 25% PWM-T
 - b) [rAc-g] 1ug, 10ug, 100ug
 - c) 6 day incubation
- 4) Condition D
 - a) 50% PWM-T
 - b) [rAc-g] 100ng, 500ng, 1ug, 5ug, 10ug
 - c) harvest well supernatant on days 4, 5, 6 & 7

The data suggests there is no major difference between 25% and 50% PWM-T cell supernatant. Best results are at 6 day incubation; day 4 & 5 yield suboptimal results. Antibody activity, cell viability decreases beyond 7 days.

No major differences were observed between glycosylated and non-glycosylated ricin A-chain forms. (Data suggests immunoreactive rAc epitopes are protein and not carbohydrate.)

Isotopes of Human Immune Response from IVI Protocol

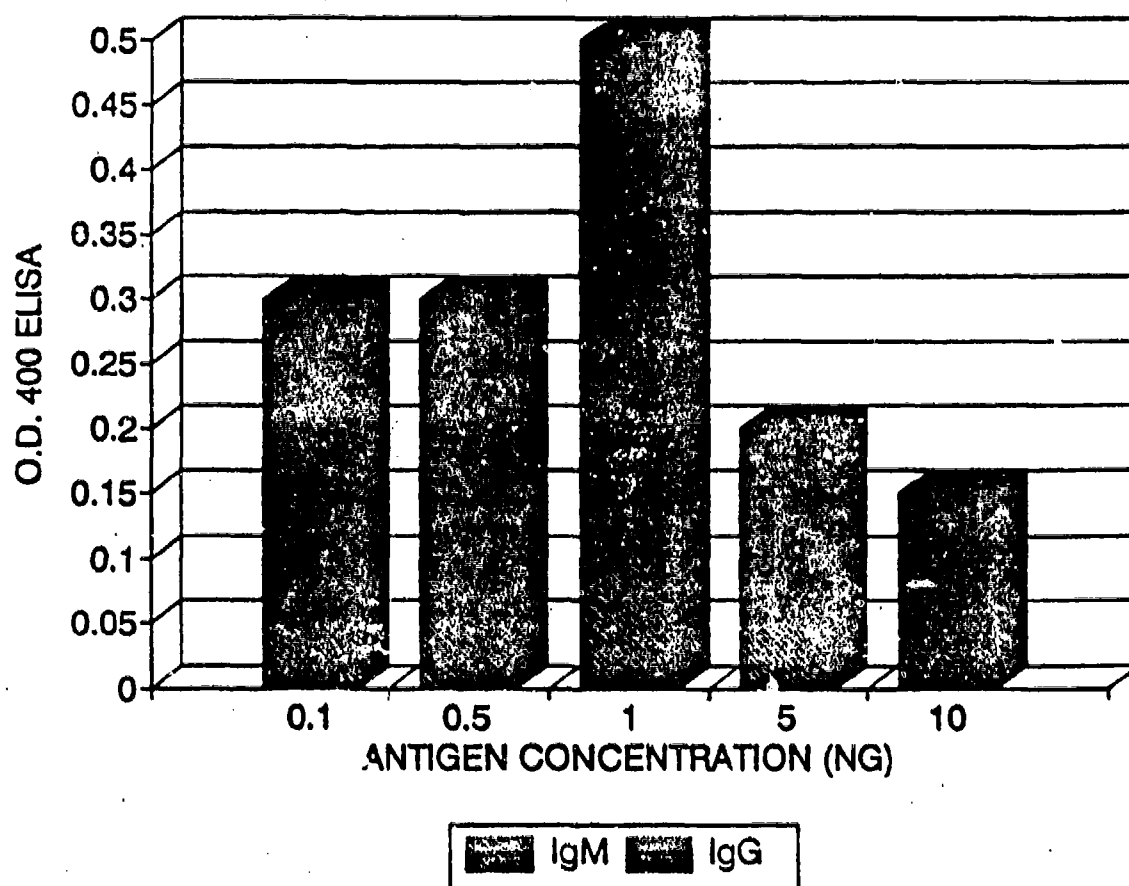
<u>Spleenocyte Source</u>	<u>Protocol</u>	<u>Immunoglobulin Isotope, %</u>	
		<u>IgM</u>	<u>IgG</u>
Patient 1	IVI	33%	11%
	no IVI	0%	0%
Patient 2	IVI	44%	0%
	no IVI	11%	0%
Patient 3	IVI	44%	0%
		11%	0%

*[(number of specific isotype-secreting, immunoreactive wells)/(number of wells plated)] x 100

<u>Experimental Condition</u>	<u>Reactivity (IgM)^a</u>			<u>Reactivity (IgG)^a</u>		
	<u>rAc-g^a</u>	<u>rAc-ng^a</u>	<u>rAc-p^a</u>	<u>rAc-g^a</u>	<u>rAc-ng^a</u>	<u>rAc-p^a</u>
exp 1	-	-	-	-	-	-
2	+	+	-	-	-	-
3	+	+	+	-	-	-
4	+	+	+	+	+	-
5	-	-	-	-	-	-

^areactive antibody is any supernatant from a 96-well plate which reacted with the target antigen in a standard ELISA ("+" ; > 2 x over background; "-" ; < 2 x background)

^ag = glycosylated
ng = nonglycosylated
p = peptide



After 6 days in culture (see Condition "B" on page 4), supernatants were harvested and analyzed by EIA for human IgM and human IgG response to rAc. This target antigen was rAc-g and rAc-ng; no difference was observed between IgM and IgG response.

Results indicate that the best concentration of rAc to use for IVI is 1ug/ml. There did not appear to be any significant difference between splenocytes from the different patients used in this experiment.

In a final experiment, spleenocytes from patient #1 were used for immortalization. The experimental procedure was fused with SHFP-1 using standard hybridoma technology. After approximately 14 days post-fusion, supernatants from developed hybridomas were screened against rAc by ELISA. Though there were IgM secreting hybridomas, the reactivity of these MAb's to rAc was weak.

Fusion Summary:

	<u>Patient 1*</u>	<u>no-IVI Control*</u>
# of clones/# wells plated (%)	39/192 (20%)	4/48 (8%)
#Ig isotope clones	IgM: 35 IgG: 4	IgM: 4 IgG: 0
rAc reactive clones	8 ^a	0

*IVI protocol for this patient's spleenocytes was 'Condition B'

*patient's spleenocytes used as a control with no IVI setup

^asupernatants from 8 hybridomas showed weak reactivity by ELISA to rAc. During expansion of clones, antibody activity was lost. [Possibility exists that these hybridomas may have recognized EIA plates and/or FCS antigens.]

CONCLUSIONS

This SBIR project was broken down into two separate phases. The first phase was to demonstrate that a human immune response could be generated to the A-chain of ricin. The second phase centered on the immortalization of this immune response.

Two unanticipated problems were encountered. The first was the high percentage of non-specific binding to EIA plates. In essence, culture supernatants showed excessive reactivity to wells of 96-well plates which did not receive any rAc antigen. (Note: the plastic of 96-well plates, polystyrene, has exposed phenyl rings which evidently share common epitope structure with most proteins (such as PHE & TYR residues) creating non-specific binding; this was overcome by excessive blocking with albumin and tween-80).

The second problem involved the recognition of the protein antigens naturally present in fetal calf serum. FCS is necessary for spleenocyte growth and proliferation. Most of the generated IVI immune response was focused on the proteins present in FCS. This can be somewhat overcome by dosing out the target antigen whereby the immune response to FCS antigens is minimized.

We conclude that it was possible to observe a human immune response to ricin A-Chain using in vitro immunization protocols. There is variability in the response of different patient's spleenocytes to IVI/rAc protocols. We suggest that, to further improve the human immune response to ricin A-chain, different adjuvant-like compounds should be used. (e.g., rAc coupled to a highly immunogenic macromolecule)

In these experiments, the predominant immune response generated was of the IgM type. We suggest that additional patients, the use of adjuvant-like compounds discussed above, or variations in the screening protocols should allow the isolation of IgG specific clones.